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Association of a single nucleotide polymorphism in *Tbx4* with developmental dysplasia of the hip: a case-control study

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SUMMARY

Objective: Developmental dysplasia of the hip (DDH), formerly known as congenital dislocation of the hip, comprises a spectrum of abnormalities, including abnormal acetabular shape (dysplasia) and malposition of the femoral head during embryonic, fetal and infantile growth periods. Genetic factors play a considerable role in the pathogenesis of DDH. As a key regulator for the hindlimb outgrowth and identification, *Tbx4* may be involved in the aetiology and pathogenesis of DDH. Our objective is to evaluate whether the *Tbx4* (rs3744438 and rs3744448) single nucleotide polymorphisms (SNPs) are associated with DDH in Chinese.

Method: The *Tbx4* SNPs were genotyped in 505 children with DDH and 551 control subjects and their association was evaluated statistically.

Results: Rs3744438 was not associated with DDH. Rs3744448 was significantly associated with DDH in the dominant genetic model of males ($P = 0.039$; odds ratio (OR) = 0.56; 95% confidence interval (CI) = 0.32–0.97) and allele G was significantly lower in patients than controls compared with allele C ($P = 0.02$; OR = 0.59; 95% CI = 0.37–0.92). After adjusted for gender, we discovered a significant association with hip dislocation in the dominant genetic model when stratified by severity ($P = 0.03$; OR = 0.73; 95% CI = 0.55–0.97), but not with subluxation and instability.

Conclusions: Tbx4 tends to play an important role in the aetiology of DDH.

Introduction

Developmental dysplasia of the hip (DDH), formerly known as congenital dislocation of the hip, comprises a spectrum of abnormalities, including abnormal acetabular shape (dysplasia) and malposition of the femoral head during embryonic, fetal and infantile growth periods[1,2]. The soft tissue capsule of the hip joint is excessively lax and allows the femoral head to displace from the confines of the acetabulum[3]. The displacement of the femoral head may be only partial subluxation or may be complete dislocation.

Most developed countries reported an incidence of 1.5–20 cases of DDH per 1000 births, the variation due in part to differences in diagnostic method and timing of evaluation[4]. The incidence in China was estimated about 0.1–0.5%[5]. Although in most affected infants the problem resolves spontaneously in the first several months of life, persistent DDH may result in chronic pain, gait abnormalities and degenerative arthritis[6–8]. It was reported that many infants with DDH have some identifiable risk factors, including first-degree relative with DDH, breech delivery, female sex, multiple gestation, first pregnancy, high birth weight, oligohydramnios and clinical evidence of joint instability[9–11]. Recently, hereditary factors had been paid more attention to the development of DDH. A genome-wide screening of a Japanese family with acetabular dysplasia identified a linkage on a limited location of the specific chromosome[12]. A single nucleotide polymorphism (SNP) in growth differentiate factor 5 gene was found significantly associated with the DDH in Chinese[13].

T-box genes, originally identified in the mouse, are highly conserved among vertebrates and play a central role in formation of posterior mesoderm and axial development[14]. *Tbx4*, which is expressed throughout the limb mesenchyme during vertebrate animal embryo development, is a key regulator for the hindlimb outgrowth and identification[15,16]. Human *Tbx4* gene, with 27857bp

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in size, located on 17q21-q22 and mutations in human *Tbx4* gene will cause small patella syndrome (SPS; OMIM 147891), which is characterized by patellar aplasia or hypoplasia and by anomalies of the pelvis and feet, including disrupted ossification of the ischia and inferior pubic rami[17]. Limb-specific deletion of *Tbx4* mice had hypoplastic pelvis and fibula, severely hypoplastic femurs and mild or nonexistent anterior digit fusions and the hindlimbs didn't articulate with the pelvis[18].

Considering the important roles *Tbx4* played in hindlimbs growth and morphogenesis, we hypothesized that SNPs in *Tbx4* gene may individually or jointly contribute to the risk of DDH. To test this hypothesis, we checked all the SNPs causing missense mutation of *Tbx4* but only the rs3744438 and rs3744448 have the allele frequency of Chinese Han population in HapMap. Thus the two SNPs (rs3744438 and rs3744448) located in exons of the *Tbx4* gene causing missense mutation (A [Ala] to V [Val] and G [Gly] to A [Ala] respectively) were examined in a case-control study with 505 patients and 551 control subjects in a Chinese population. The two SNP were not in Linkage disequilibrium ($r^2 = 0$).

Materials and methods

Subjects

The 505 radiology confirmed DDH patients were consecutively recruited between June 2007 and October 2009 from the Center of Diagnosis and Treatment for Development dysplasia of hip, Kang'ai Hospital, China. 551 controls were randomly selected from a pool of more than 2000 individuals, who enrolled at the Physical Examination Center, Drum Tower Hospital, affiliated to the Medical School of Nanjing University, China during the same time period as the cases were recruited and had no sign of DDH.

Patients with unilateral or bilateral DDH were diagnosed by expert medical examination with radiographic evidence excluding those cases with systemic syndrome. Control subjects were identified by taking a detailed history and physical examination. The severity of DDH was defined as three grades, from mild instability of the femoral head with slight capsular laxity, to moderate lateral displacement of the femoral head, without loss of contact of the head with the acetabulum, and then to complete dislocation of the femoral head from the acetabulum[19]. Cases were scored according to the severity of the hip disorder (1 = instability; 2 = subluxation; 3 = dislocation). During the process of the study no subjects dropped out. The subjects' recruitment was approved by the ethics review committee of the Nanjing University and informed consent was obtained from patients and controls. All of the cases and control subjects were unrelated ethnic Han Chinese living from all around China.

Genotyping

DNA was obtained from all the subjects from peripheral blood using the Chelex-100 method[20] or buccal swabs using the DNA IQ System (Promega, Madison, WI) according to the manufacturer's instructions. The SNP rs3744438 and rs3744448 of case and control subjects were genotyped using Taqman assay (Applied Biosystems 7900, ABI, Foster City, CA). Genotyping was performed by laboratory personnel blinded to case status, and two authors independently reviewed the genotyping results, data entry and statistical analyses. 5% samples of case or control subjects were randomly selected to repeat, yielding a 100% concordant. The minor allele frequency of rs3744438 and rs3744448 was above 0.05 in the Chinese population respectively.

Statistics

For this case-control association study, we used standard χ^2 tests to determine the significance of differences in allelic and genotypic frequencies between DDH patients and control subjects and $P < 0.05$ was considered statistically significant. The associations between *Tbx4* variants and DDH risk were estimated by computing the odds ratios (ORs) and 95% confidence intervals (CIs) from both univariate and multivariate logistic regression analyses with adjustment for sex. The Hardy–Weinberg Equilibrium was tested by a goodness-of-fit χ^2 test to compare the observed genotype frequencies with the expected ones among the control subjects. All the statistical analyses were performed with SAS 9.1.3 software (SAS Institute, Cary, NC). The statistical power for each model was calculated using PGA software and the results were shown in Tables I and II.

Results

All of the controls and DDH cases in this study were composed of Han population. The ages of patients with DDH and controls (mean ± SD) were 21.1 ± 12.9 months (range 2–99 months) and 57.4 ± 11.2 years (range 28–97 years), respectively. The height and weight of controls were 163.7 ± 7.7 cm (range 140–184 cm) and 63.8 ± 10.7 kg (range 39–95 kg), respectively and the mean body mass index (BMI) was 23.7 ± 3.0 kg/m^2 (range 16.2–35.5 kg/m^2). Distributions of genotypes of rs3744438 and rs3744448 in controls were all conformed to Hardy–Weinberg Equilibrium ($P = 0.59$ and 0.19, respectively). The minor allele frequencies of rs3744438 and rs3744448 of controls in our study are 0.09 and 0.34 respectively and they were close to those reported in HapMap for Chinese Han populations (0.12 and 0.31 respectively). The ratio of female to male was about six to one in patients with DDH. Only 4.7% of patients were reported to have family history of DDH. 52.1% of the DDH cases were delivered by caesarean section and 8.0% of the DDH cases were premature labor. The distribution of the severity of the hip disease was 8.4% with instability, 12.5% with subluxation and 79.1% with dislocation. Details of the genotypes and allele distributions of controls and DDH cases were seen in Supplementary material Tables 1 and 2.

In the association study, no significant differences in genotype frequencies of rs3744438 between cases and controls were

Table I
Association of 3744448 of the *Tbx4* gene with DDH when stratified by gender

Groups compared	GG vs GC/CC				GG/GC vs CC				G allele vs C allele			
	P value	OR	95% CI	Power	*P* value	OR	95% CI	Power	*P* value	OR	95% CI	Power
All patients ($n = 505$) vs all controls ($n = 551$)	0.827	0.96	0.640–1.440	0.039	0.089	0.810	0.630–1.030	0.388	0.171	0.878	0.730–1.057	0.275
Female patients ($n = 422$) vs female controls ($n = 303$)	0.501	1.186	0.722–1.947	0.105	0.117	0.784	0.578–1.063	0.352	0.394	0.907	0.724–1.135	0.132
Male patients ($n = 67$) vs male controls ($n = 240$)	0.106	0.364	0.107–1.240	0.426	**0.039**	0.556	0.318–0.972	0.545	**0.020**	0.585	0.372–0.918	0.657

DDH: developmental dysplasia of the hip; CI: confidence interval; OR: odds ratio. The significance of bold in Table means that the *P* value is below 0.05.

Table II
Association of 3744448 of the *Tbx4* gene with DDH when stratified by severity (adjust for gender)

Groups compared	GG vs GC/CC				GG/GC vs CC				G allele vs C allele			
	P value	OR	95% CI	Power	*P* value	OR	95% CI	Power	*P* value	OR	95% CI	Power
Patients with hip instability ($n=41$) vs all controls ($n=551$)	0.299	0.463	0.108–1.982	0.225	0.271	0.695	0.363–1.329	0.197	0.201	0.717	0.430–1.194	0.259
Patients with hip subluxation ($n=61$) vs all controls ($n=551$)	0.382	1.439	0.636–3.256	0.138	0.178	0.683	0.393–1.189	0.279	0.554	0.881	0.580–1.339	0.088
Patients with hip dislocation ($n=387$) vs all controls ($n=551$)	0.906	0.973	0.614–1.541	0.033	**0.032**	0.732	0.550–0.974	0.636	0.100	0.837	0.677–1.034	0.408

DDH: developmental dysplasia of the hip; CI: confidence interval; OR: odds ratio. The significance of bold in Table means that the *P* value is below 0.05.

observed either in allele or genotype frequencies even when stratified by gender or severity (data not shown). In SNP rs3744448, we still didn't find any significant difference in any comparison as a whole. When stratified by gender, we observed significant differences in the dominant model (GG/GC vs CC) of male subjects ($P=0.039$) (Table I). We could also observe that significant differences in allele frequency between DDH and control groups in male subjects ($P=0.020$) (Table I). No significant difference was detected in the comparison of genotype and allele frequency between female DDH and control subjects. We could observe that the genotype distribution and allele frequency in male members of the DDH were different to that in the female samples and male or female controls. The G allele was significantly lower in DDH male subjects compared with controls (Supplementary Table 1). In rs3744448 polymorphism, no significant difference was found between samples with hip dislocation, instability and subluxation when stratified by severity but after adjustment for gender a significant difference was found in the dominant model (GG/GC vs CC) between samples with hip dislocation ($P=0.03$) (Table II).

Discussion

It was demonstrated that ablation exon5 of *Tbx4* would cause the hypoplastic pelvis and fibula, severely hypoplastic femurs in mice and it also appeared that the mice hip joint was dislocated[18]. All these suggest that *Tbx4* may play an important role in the development of the hindlimb and may be the disease-causing gene of DDH. The functional study had interested us in assessing whether the genetic polymorphisms in *Tbx4* were associated with DDH in Chinese population[18]. However, we only found a weak association between the male DDH cases and the male controls in rs3744448. To date, this is the first demonstration of an association between *Tbx4* and DDH in Chinese, although this association only existed in the dominant model (GG/GC vs CC) of male subjects and was near the threshold (Table I). When compared G allele with C allele, the association also reached significance (Table I). Thus, it is suggested that the G allele may play a protective role against C allele in male subjects. After adjusted for gender, we discovered the significant association with hip dislocation when stratified by severity (Table II), but not with subluxation and instability. This indicates that the SNP may be associated with severity of DDH. Future research should be conducted with a larger sample number to clarify this finding. All of the statistical powers data were above 54% in the genetic model that we found have statistical difference. Based on the sample size, these power data support our finding here.

There is no significant association of rs3744438 in any comparison of DDH and control subjects. One possible explanation is that our study has relatively limited sample numbers, especially with male DDH subjects, preventing us from finding potential association between DDH and *Tbx4*. The second possible reason is that DDH may be associated with other loci than rs3744438 within the *Tbx4* in our study. Since a lot of SNPs in *Tbx4* haven't been studied in our study and many of them even don't have genotype or allele frequency on PubMed or HapMap Project, further work should be done to evaluate the possible association between *Tbx4* and DDH in Chinese or Caucasian in other SNPs. Since DDH is a multigene disorder, many susceptible genes may contribute to the occurrence of DDH[21]. Still another possibility is that the major disease-causing genes are not *Tbx4*. Our study team has already confirmed the association between *GDF5* and DDH[13]. A UK group finds possible association between Vitamin D and oestrogen receptor polymorphisms and DDH[22]. Another group of China focused on the association between the polymorphisms of PCOL2 and Sp1 binding sites of *COL1A1* gene and DDH in a Chinese population, but found negative result[23]. In future other genes involved in the development of DDH need to be further studied.

The function of *Tbx4* is still not well known. *Tbx4* is crucial for the development of the hindlimb, but it also involved in the development of other organ like heart and lung[24,25]. Recently, it is reported that a *Tbx4* polymorphism may contribute to the susceptibility of breast cancer and involved in the detection of biomarkers in pancreatic ductal adenocarcinomas[26,27]. Further works need to be done to clarify its definite function in development and its susceptibility to cancer.

There are several limitations in our study. One obvious is that DDH affects more females than males, thus the number of male DDH subjects is relatively limited in our study. It is necessary to collect large numbers of subjects to confirm or refute our findings in male subjects. The second is that our study is a hospital-based case-control study, using the cases and controls from hospitals. The study subjects may not be representative for the target population. Thirdly, after Bonferroni corrections only one association (G allele vs C allele between male patients and male controls of *Tbx4* polymorphism rs3744448) were still significant with the significance level of 0.025 (0.05/2). False positive report probability (FPRP) analysis revealed that all positive findings except for GG/GC vs CC between male patients and male controls remained significant assigning prior probability above 0.1, but the associations may be less convincing setting the prior probability of 0.01, when considering 0.5 as the FPRP level criterion based on the potential functional significance of the selected SNPs[28]. Therefore large well-designed studies with diverse populations and functional characteristics are warranted to confirm or refute our findings in future. However, our study has several strengths. The multivariate logistic regression analyses and stratified analyses were used to adjust the confounding factors and the rigorous laboratory work was conducted to ensure the veracity of genotyping data.

In conclusion, the present study did not find any significant association between rs3744438 and susceptibility to DDH in the Chinese population. But, we found that rs3744448 is associated with DDH in dominant model of male subjects or patients with hip dislocation when stratified by severity after adjustment for gender.

Authors' contributions

Study coordination and responsibility for the integrity of the work: Qing Jiang.

Conception and design: Kejie Wang, Dongquan Shi.

Analysis and interpretation of the data: Kejie Wang.

Drafting of the article: Kejie Wang, Dongquan Shi.

Critical revision of the article for important intellectual content: Dongquan Shi, Qing Jiang.

Final approval of the article: Qing Jiang.

Provision of study materials or patients: Pengsheng Zhu, Hongtao Zhu, Baocheng Zhao, Jin Dai, Lunqing Zhu, Kejie Wang, Yanyun Lv.

Statistical expertise: Kejie Wang.

Obtaining of funding: Qing Jiang.

Administrative, technical, or logistic support: Dongquan Shi.

Collection and assembly of data: Jin Dai, Lunqing Zhu, Kejie Wang, Yanyun Lv.

Conflict of interest

The authors declare that they have no competing interests.

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Supplementary data

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.joca.2010.09.008.

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